The digestive tract and derived primordia differentiate by following a precise timeline in human embryos between Carnegie stages 11 and 13

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Abstract

The precise mechanisms through which the digestive tract develops during the somite stage remain undefined. In this study, we examined the morphology and precise timeline of differentiation of digestive tract-derived primordia in human somite-stage embryos. We selected 37 human embryos at Carnegie Stage (CS) 11–CS13 (28–33 days after fertilization) and three-dimensionally analyzed the morphology and positioning of the digestive tract and derived primordia in all samples, using images reconstructed from histological serial sections. The digestive tract was initially formed by a narrowing of the yolk sac, and then several derived primordia such as the pharynx, lung, stomach, liver, and dorsal pancreas primordia differentiated during CS12 (21–29 somites) and CS13 (≥ 30 somites). The differentiation of four pairs of pharyngeal pouches was complete in all CS13 embryos. The respiratory primordium was recognized in ≥ 26-somite embryos and it flattened and then branched at CS13. The trachea formed and then elongated in ≥ 35-somite embryos. The stomach adopted a spindle shape in all ≥ 34-somite embryos, and the liver bud was recognized in ≥ 27-somite embryos. The dorsal pancreas appeared as definitive buddings in all but three CS13 embryos, and around these buddings, the small intestine bent in ≥ 33-somite embryos. In ≥ 35-somite embryos, the small intestine rotated around the cranial-caudal axis and had begun to form a primitive intestinal loop, which led to umbilical herniation. These data indicate that the digestive tract and derived primordia differentiate by following a precise timeline and exhibit limited individual variations.

Key Words: Timeline, digestive tract, derived primordia, human embryo, development

Introduction

During development, all vertebrates pass through the somite stage, which is defined as any developmental stage between the formation of the first and last pairs of somites in embryos (Gilbert, 2003; Müller and O'Rahilly, 2003). While the craniocaudal axis of the body is formed immediately before the beginning of the somite stage, almost all major organ primordia are detected at the end of the somite stage. Moreover, together with digestive-tract derivatives, the digestive tract, which includes the foregut, midgut, and hindgut, forms one of the main organ systems during the somite stage. The pharynx, endocrine organs, lung, liver, pancreas, gall bladder, cloaca, and allantoic duct differentiate from the digestive tract as primordia during the somite stage.

The developmental staging system used for human embryos is called the Carnegie Stage (CS) system; in this system, embryos are categorized based on internal and external characteristics (O'Rahilly and Müller, 1987, 2010). The somite stages correspond to CS9–CS13 and occur approximately in the third and fourth weeks after the fertilization of the ovum. As the number of pairs of somites increases, embryo development becomes more dynamic and obvious. Moreover, the digestive tract and derived primordia form rapidly in only 2 days during CS12 (21–29 somites; approximately 29–31 days after fertilization) and then differentiate and grow during CS13 (≥ 30 somites; 30–33 days after fertilization). Detailed studies conducted on CS12 embryos have primarily analyzed histological sections obtained from case studies. For example, 23- (Thompson, 1907), 25- (West, 1937), and 27-somite embryos (Waterson, 1914) have been precisely described. The external and internal morphologies differ between these embryos and characterize CS12 (O'Rahilly and Müller, 1987). The number of somites is not used as an indicator of the timing of differentiation in CS13 because somite counting is not sufficiently accurate, and the final number of somites in each embryo varies among the embryos of this stage. Moreover, no studies have clarified the dynamic temporal changes in the digestive tract and the differentiation of derived primordia in human somite-stage embryos at CS12 and CS13.

Therefore, we designed this study to establish the precise timeline of the formation of the digestive tract and derived primordia in somite-stage human embryos. Three-dimensional (3D) reconstructions demonstrated a dynamic and synchronized formation of this organ system, which differentiated according to a strict timeline.

Materials and Methods

Human embryo specimens

Approximately 44,000 human embryos comprising the Kyoto Collection are stored at the Congenital Anomaly Research Center of Kyoto University (Nishimura et al., 1968; Shiota, 1991; Yamada et al., 2004). Most of these embryos were obtained when pregnancy was terminated during the first trimester for socioeconomic reasons, under the Maternity Protection Law of Japan. Some of the specimens (~20%) were undamaged, well-preserved embryos. When the aborted embryos were brought to the laboratory, they were measured, examined, and staged using the criteria provided by O'Rahilly and Müller (1987). Well-preserved human embryos that were found to be externally normal were subjected to histological serial sectioning. For this study, 37 human embryos were selected (one sample for CS11, 11 samples for CS12, and 22 for CS13 from the Kyoto Collection, and two samples for CS12 and one for CS13 from the Blechschmidt Collection) (Blechschmidt, 1961). All of these samples exhibited no overt damage or anomalies, and the histological conditions were well preserved.

Histological observations

Histological serial sections (transverse sections, 10-μm thick) of whole embryos were scanned and stored as digital data by using a film scanner (CanoScan 9000F, Canon, Tokyo, Japan) at 4800 dpi. The following primordia were histologically observed in digital images: the epithelium and mesenchyme of thyroid, the epithelium around the respiratory primordium region, omental bursa, ventral pancreas, and liver parenchyma.

3D reconstruction of the digestive tract and derived primordia

Sequential 2D images were trimmed digitally using ImageJ64 (ver. 1.46, National Institutes of Health, Bethesda, MD, USA) and saved as Microsoft Windows Bitmap Images (.bmp files) at a resolution identical to that of the original digital data. The inner walls of the digestive tract and the primordia and each somite were segmented, and 3D images were computationally reconstructed for examining the morphology in all samples in Amira software (ver. 5.5.0, Visage Imaging, Berlin, Germany). Alignment was manually adjusted based on the external 3D view of the segmented digestive tract and primordia, using external images as a reference (Fig. 1). The beginning and end of the stomach and the edge of the yolk sac/stalk were identified using the inflection point of the 3D morphology and the cross-sectional view of each sample. Conversely, the

level of each primordium according to somite number was determined based on the 3D relationship between somites and the respective primordia. The first somite was determined carefully based on its relationship with key anatomical references, such as rhombomeres 1–8, pharyngeal arches 1–4, and the otic vesicle (Müller and O'Rahilly, 2003). The longitudinal length of the connection of the yolk sac and trachea was measured in Amira.

Results

Morphology of the digestive tract

Whole embryos were 3D reconstructed after segmentation of the digestive tract and derived primordia (Fig. 1). The digestive tract and derived primordia were then examined in 3D to analyze their morphology and position within the body. The stalk of the yolk sac was broad (measuring approximately 1500 μm in longitudinal length), and the primitive digestive tract was not differentiated into any derived primordia in the 16-somite embryo at CS11 (data not shown). The main primordia, including the pharynx, the respiratory primordium as a single protrusion, liver, yolk sac, allantoic duct, and cloaca, were detectable during CS12 and later stages (Fig. 1A). The stomach, dorsal pancreas, and gall bladder were detectable during CS13 (Fig. 1B).

Pharynx and pharyngeal pouches

The pharyngeal region of the digestive tract was a simple tubular structure in one of four 21-somite embryos. The digestive tract developed in the transverse direction and became thin (flat in three out of four 21-somite embryos and two 22-somite embryos), and three pairs of pharyngeal pouches were detected in one out of two 22-somite embryo and all 23–29-somite embryos. The pharyngeal pouches became comparatively deeper and distinct in embryos at CS13 (≥ 30-somite embryos; Fig. 2A). No sign of the oropharyngeal membranes was detected in any of the samples used in this study.

The luminal projection of the digestive tract on the mid-sagittal plane between the first and second pharyngeal pouches was observed for a limited time in one of two 27-somite embryos, one 29-somite embryo, and one 30-somite embryo ("lp" in transverse section b of Fig. 2A and in Fig. 2B-a). The minute vessel, which is linked to truncus arteriosus, was present in the central part of the mesenchyme of the round prominence. The thyroid diverticulum was formed at the location of this projection in ≥ 31-somite embryos at CS13 ("th" in Fig. 2B-b).

Esophagus and stomach

The digestive tract at the region of the esophagus and stomach was thick in the ventral-dorsal direction but was not recognizable as definite esophagus and stomach primordia in the 3D images of any of the CS12 embryos. In histological sections, the region around the digestive tract appeared symmetrical at the mid-sagittal plane in embryos containing ≤ 23 somites (Fig. 2B-c). In serial sections, the epithelium of the

ventral side appeared more columnar than did the epithelium of the dorsal side. In embryos of later stages, the respiratory primordium appeared from the ventral side. The region rotated slightly, and the omental bursa was observed in all embryos containing ≥ 24 somites ("ob" in Fig. 2B-d). A spindle-shaped stomach was formed in one of three 32-somite embryos, one of two 33-somite embryos, and all ≥ 34-somite embryos (Fig. 2A).

Small intestine

The yolk sac was wide open (Fig. 1A). The digestive tract around the liver bud was not curved during CS12 but bent gradually during CS13. The digestive tract bent in the ventral direction with the emergence of the dorsal pancreas primordium in all ≥ 33-somite embryos at CS13. Moreover, the intestine began to rotate around the cranial-caudal axis (body axis), and this occurred most markedly around the region of the pancreas primordia in all ≥ 35-somite embryos. The connection between the yolk sac and the digestive tract became constricted and formed the yolk stalk, and the formation of the primitive intestinal loop was then initiated in all ≥ 35-somite embryos. The connection of the yolk sac was measured longitudinally (Fig. 3); it was 1500-μm long in 16-somite embryos, and then it shortened and became < 130-μm long in ≥ 31-somite embryos.

Cloaca

The cloaca was bag-shaped and appeared convex in transverse sections of 21–26-somite embryos (Fig. 2A). The gut caudal of the cloaca membrane (tail gut) became gradually evident beginning in 26-somite embryos. The transverse width decreased in embryos containing ≥ 27 somites. The dorsal side of the cloaca increased in diameter, and the prospective urorectal cleavage line appeared. The caudal tip of the cloaca appeared sharp in two of five 35-somite embryos and in all ≥ 36-somite embryos at CS13. The cloacal membranes were observed in all samples examined in this study.

Morphology of the digestive tract-derived primordia

Respiratory primordium (trachea and bifurcation)

The respiratory primordium was not recognized in the 3D images of any 21–25-somite embryos, but was observed as a high columnar epithelium in serial sections ("rp" in Fig. 4B-a). The respiratory primordium was recognized as a single protrusion for the first time in the 26-somite embryo and was clearly observed in the 29-somite embryo shown in Fig. 4A. The respiratory primordium flattened out

transversely and became the primitive form of the bifurcation in 30–34-somite embryos at CS13 (“bi” in Fig. 4A). Concurrently, the trachea was formed and, consequently, the separation point between the trachea and the esophagus could be detected in all ≥ 35-somite embryos at CS13 (* in Fig. 4A). The length of the trachea then increased, and this indicated the separation from the digestive tract and a caudal descend of the bifurcation. The distance between the separation point and the bifurcation was approximately equal to the length of two somites (520 μm on average) in ≥ 36-somite embryos.

Pancreas

The primordium of the dorsal pancreas appeared in the 3D images as a definitive budding in all embryos, but it was detected as a primitive protrusion in three embryos at CS13 (Fig. 4A). The ventral pancreas could not be recognized in the 3D images of any of the embryos at CS13 but was observed as a high columnar epithelium in a few serial sections (“vp” in Fig. 4B-b) in seven of 13 embryos containing ≥ 35 somites.

Liver and gall bladder

The liver diverticulum was not recognizable in the 16-somite embryo at CS11, but was formed in the cranial side of the opening of the yolk sac in all 21-somite embryos at CS12. The liver bud was formed in 27-somite embryos and was clearly detectable in the 29-somite embryo (Fig. 4A). The liver diverticulum was detected as a rounded evagination, and a high columnar epithelium was observed in histological sections during CS12 (Fig. 4B-c). Hepatic cells were arranged in a trabecular form in six of nine 30–34-somite embryos at CS13 (Fig. 4B-d), whereas the border of the liver parenchyma was clearly recognized in all ≥ 35-somite embryos at CS13 (Fig. 4B-e).

The gall bladder was recognized as a protrusion for the first time in 30–34-somite embryos and was formed in all ≥ 35-somite embryos at CS13.

Position of the digestive tract-derived primordia along the craniocaudal axis

When the craniocaudal axis elongated, the number of pairs of somites and the length of the digestive tract increased concomitantly. The connection between the yolk sac and the digestive tract became confined during CS12 (Fig. 3). However, the length of each somite around the yolk sac in 21–32-somite embryos remained relatively constant at 90–130 μm.

Each digestive tract-derived primordium descended caudally (Fig. 5). By

contrast, the respiratory primordium did not descend after it was recognized as a single protrusion around the level of the third and fourth somites (Fig. 6), which agrees with the results of a previous study (O'Rahilly and Müller, 1984a). The respiratory primordium and the liver bud were located near each other in 21–22-somite embryos and were separated by a distance of only one somite (approximately 100 μm). In ≥ 23-somite embryos, the primitive stomach was formed between the liver bud and the respiratory primordium.

The stomach, dorsal pancreas, and liver primordia descended caudally at CS12 and established their definitive positions at CS13 (Figs. 5 and 6). The stomach, dorsal pancreas, and liver were located close to each other and maintained the same relative position within the length of one somite during CS13.

The position of the separation point of the trachea and the esophagus along the craniocaudal axis showed limited variation, and this point remained at the level of the fourth somite in all samples at CS13. However, the positions of the stomach, lungs, dorsal pancreas, and liver primordia showed variations of one and three somites along the craniocaudal axis during CS13 (Fig. 6).

The allantoic duct and cloaca descended caudally and continuously during CS12, and their positions varied considerably along the craniocaudal axis between embryos containing identical numbers of somites at CS13.

Discussion

In this study, the differentiation of the digestive tract and derived primordia during human somite stages CS11–CS13 was visualized in 3D by using images reconstructed from digitalized serial histological sections. Our data allowed us to create a precise timeline of the differentiation events and provided key insights into these developmental processes. The major differentiation events are described in Figure 7.

The connection of the yolk sac was broad in 16-somite embryos, but was then constricted until embryos reached the 32-somite stage. Although the size of umbilical ring remains constant (Vermeij-Keers et al., 1996), the internal components of the ring, such as the umbilical arteries and vein and the allantoic duct, may change during this period. The preparation for physiological umbilical herniation might also proceed latently, because the development of the small intestine was found to proceed earlier than that described previously (O'Rahilly and Müller, 1987): In the present study, in ≥ 35-somite embryos, the formation of the primitive intestinal loops was initiated. The bending and rotation along the body axis of the intestine at the region of the pancreas primordia were also initiated. These data were inconsistent with those of previous studies (O'Rahilly, 1978; O'Rahilly and Müller, 1987), which described the ventral bowing and slight deflection from median plane of the intestine during initiation of the primitive intestinal loop at CS14.

The 3D data presented herein clarified the position and morphological changes of each primordium and further showed that the differentiation processes were closely synchronized with the described timeline. The change in the position of the primordia along the long axis around the pharynx was similar to that observed in previous studies (O'Rahilly and Müller, 1984a; Müller and O'Rahilly, 2003); we found that the first somite was caudal to the fourth pharyngeal arch and that the separation point between the trachea and the esophagus remained at the same somitic level from CS13 to CS19. Moreover, the respiratory primordium was at the level of the fourth somite at the 27-somite stage (Waterson, 1914). The primordia at the thoracicoabdominal region descended caudally during CS12, but mostly established a definitive position during CS13. In contrast, the primordia of the urinary system continued to descend during CS13, suggesting that the position of these primordia would be established during later stages. These data suggested that the position of the primordia was established definitively from the cranial to caudal direction.

The luminal projection on the mid-sagittal plane between the first and second pharyngeal pouches was observed during the limited period examined in this study. This unique phenomenon was reported previously as a variation of the thyroid primordium,

albeit without sufficient evidence to definitively support the observation (Sgalitzer, 1941; Orts-Llorca and Genis Galvez, 1958). Our results showed that the projection occurred in embryos between 27- and 30-somite stages. In ≥ 31-somite embryos, the epithelium of the thyroid primordium became thick and detached from the pharynx.

We also made histological observations on the lung, pancreas, and liver primordia, because the differentiation of the epithelium was observed in 2D histological sections before the change in the lumen in these primordia. The histological differentiation of the epithelium preceded that of the lumen of the digestive tract in the lung and ventral pancreas. We also observed a dynamic differentiation of the mesenchymal tissue, which occurred together with slight changes in the lumen in the liver. The reconstructed 3D images obtained in this study could be used for developing the morphogenesis timeline and analyzing the approximate form, relative position, and change of each primordium. However, these reconstructions lacked information regarding the tissues surrounding the examined primordia. Thus, additional studies must be conducted to elucidate the development of each organ within the interactive context of all tissue components.

Previous morphometrical studies showed that size and proportion varied considerably among embryos at the same stage (O'Rahilly and Müller, 1984b; Otani et al., 2008). In the case of somite-stage embryos (CS11–CS13), the definitive morphology of each organ could not be readily established because the morphology changed drastically within the same stage during development. For example, 13 distinct illustrations of the respiratory primordium were presented for CS13 embryos (O'Rahilly and Müller, 1987). Such information could lead to the inaccurate conclusion that the development of the human embryo does not follow a strict timeline. By contrast, the results of this study—obtained after sorting embryos according to the number of somites—demonstrated a dynamic and synchronized formation of the examined organ system, which differentiated according to a strict timeline and exhibited minimal individual differences. Furthermore, the timeline presented herein is a useful tool for detecting abnormalities in somite-stage embryos, and this might potentially allow prenatal diagnosis to be performed in such early-stage embryos in the future.

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Figure legends

Figure 1. Representative gross views of embryos and three-dimensional reconstruction of identical embryos from histological serial sections.
(A) Twenty-seven-somite embryo at CS12 (#4310)
(B) Thirty-five-somite embryo at CS13 (#1556)
The digestive tract and derived primordia (purple) were identified using the translucent silhouette of the whole embryo. ph, pharynx; rp, respiratory primordium as a single protrusion; tr, trachea; bi, bifurcation; li, liver; ys, yolk sac; ad, allantoic duct; cl, cloaca; st, stomach; dp, dorsal pancreas; gb, gall bladder.

Figure 2. Morphology of the digestive tract during CS12 and CS13.
(A) Three-dimensional morphogenesis
anterior, anterior view; transverse, transverse section at the position of the dotted line in the anterior view; left lateral, left lateral view; Arabic numbers (1–4), the number pairs of the pharyngeal pouches; lp, luminal projection; tr, trachea; es, esophagus; st+, the region in which the primordium of the stomach would be formed. cm, cloaca membrane The scale bar indicates 100 μm.
(B) Histology as shown using hematoxylin and eosin staining (200×)
ph, pharynx; lp, luminal projection; th, thyroid; dt, digestive tract; ob, omental bursa. The scale bar indicates 100 μm.
a) A projection in a 29-somite embryo (#1845). The projection was found at the location where the thyroid diverticulum forms in in embryos with more than 31 somites.
b) Thyroid in a 37-somite embryo (#2350).
c) Esophageo-gastric region in a 23-somite embryo (#589). The lumen was symmetrical at the mid-sagittal plane. The epithelium of the ventral side appeared more columnar than that of the dorsal side in serial sections.
d) Esophageo-gastric region containing the omental bursa in a 29-somite embryo (#1845). The stomach was pyramidal in shape and slightly rotated clockwise.

Figure 3. Longitudinal length of the connection of the yolk sac in embryos having 16–32 somites.

Figure 4. Morphology of digestive tract-derived primordia during CS12 and CS13.
(A) Three-dimensional morphogenesis

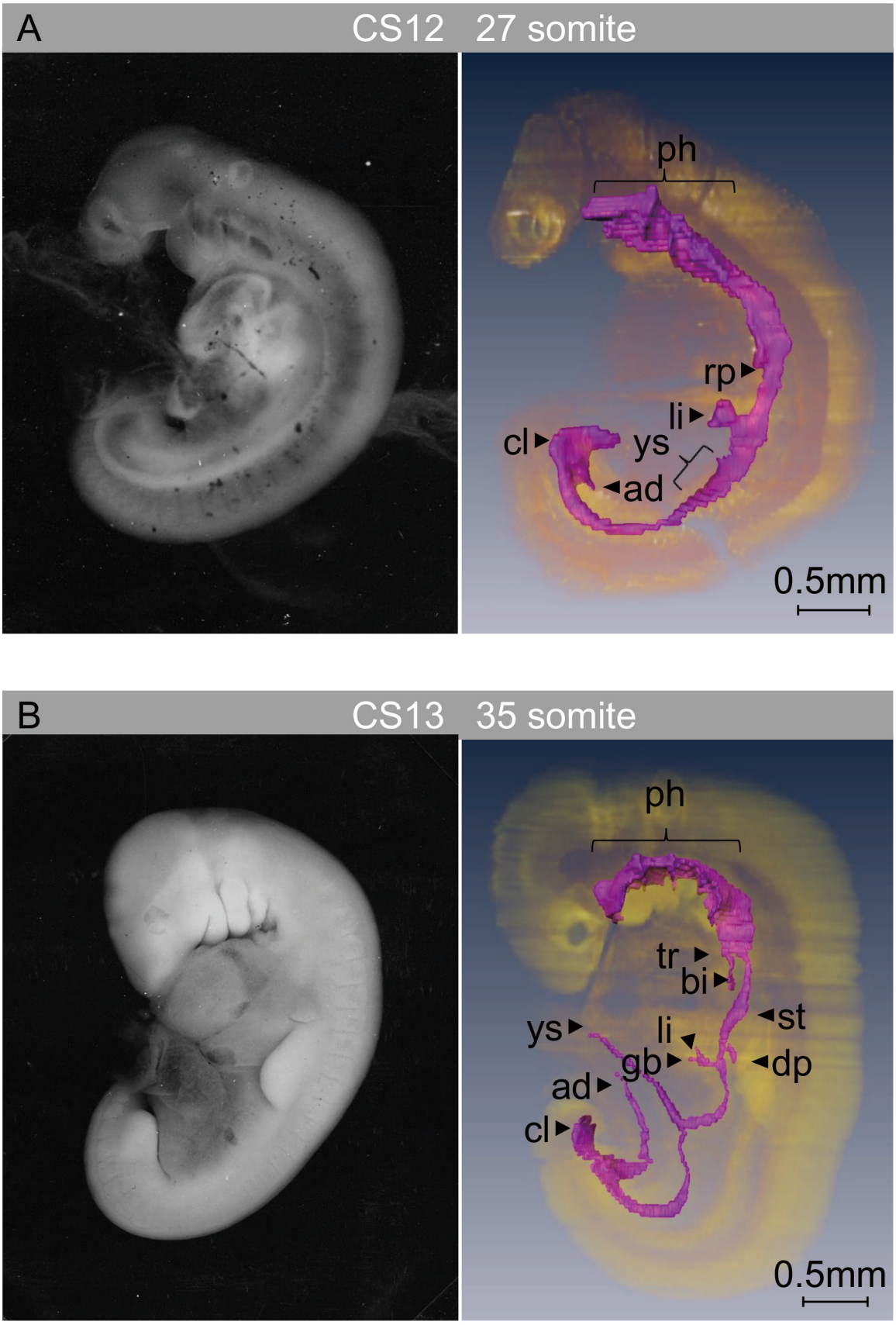
left lateral, left lateral view; ventral, frontal view; rp, respiratory primordium; tr, trachea; bi, bifurcation; es, esophagus; dp, dorsal pancreas; li, liver; gb, gall bladder; *, separation point between the trachea and the esophagus.
The scale bar indicates 100 μm.
(B) Histology, as shown using hematoxylin and eosin staining (200×)
dt, digestive tract; rp, respiratory primordium; dp, dorsal pancreas; vp, ventral pancreas; hd, hepatic diverticulum; ha; hepatic antrum. The scale bar indicates 100 μm.
a) Respiratory primordium in a 21-somite embryo (#2694). The high columnar epithelium of the digestive tract was observed on the ventral side.
b) Ventral pancreas in a 37-somite embryo (#2350).
c) Liver bud in a 21-somite embryo (#2694). The respiratory primordium and the liver bud were located near each other. The characteristics of the digestive tract were the same as those shown in (b)
d) Liver bud in a 32-somite embryo (#251). The hepatic cells were arranged in a trabecular form.
e) Liver bud in a 37-somite embryo (#2350). The border that forms the liver parenchyma was clearly recognized.

Figure 5. Position of digestive tract-derived primordia along the craniocaudal axis, shown with the number of pairs of somites used as a reference.
Arabic numbers indicate the numbers of somites. Reference points for each primordium were defined as follows: Respiratory primordium (hs*), the primordium observed as a high columnar epithelium; Respiratory/trachea (3D*), the top of the respiratory primordium detected as a single protrusion (before the trachea differentiated)/the separation point between the trachea and the esophagus; Bifurcation, the first bifurcation of bronchi; Omental bursa, the caudal point of the omental bursa (CS12); Stomach, the gastric cardia; Dorsal pancreas, the top of the protrusion or the budding point; Liver and Gall bladder, the budding point from the digestive tract (the liver and gall bladder budded from the same point); Yolk stalk, the joint with the digestive tract; Allantoic duct; Cloaca, the part containing the most cranial side of the primordium.

Figure 6. Distribution of the position of each primordium along the craniocaudal axis at CS13.
The position along the craniocaudal axis is indicated as the number of somites, and the distribution of the position is indicated as the percentage (%) at CS13, with gray color being used for comparison with CS12. The reference points used for each primordium

were the same as those in Figure 5.

Figure 7. Timeline of the development of digestive tract-derived primordia in somite-stage embryos.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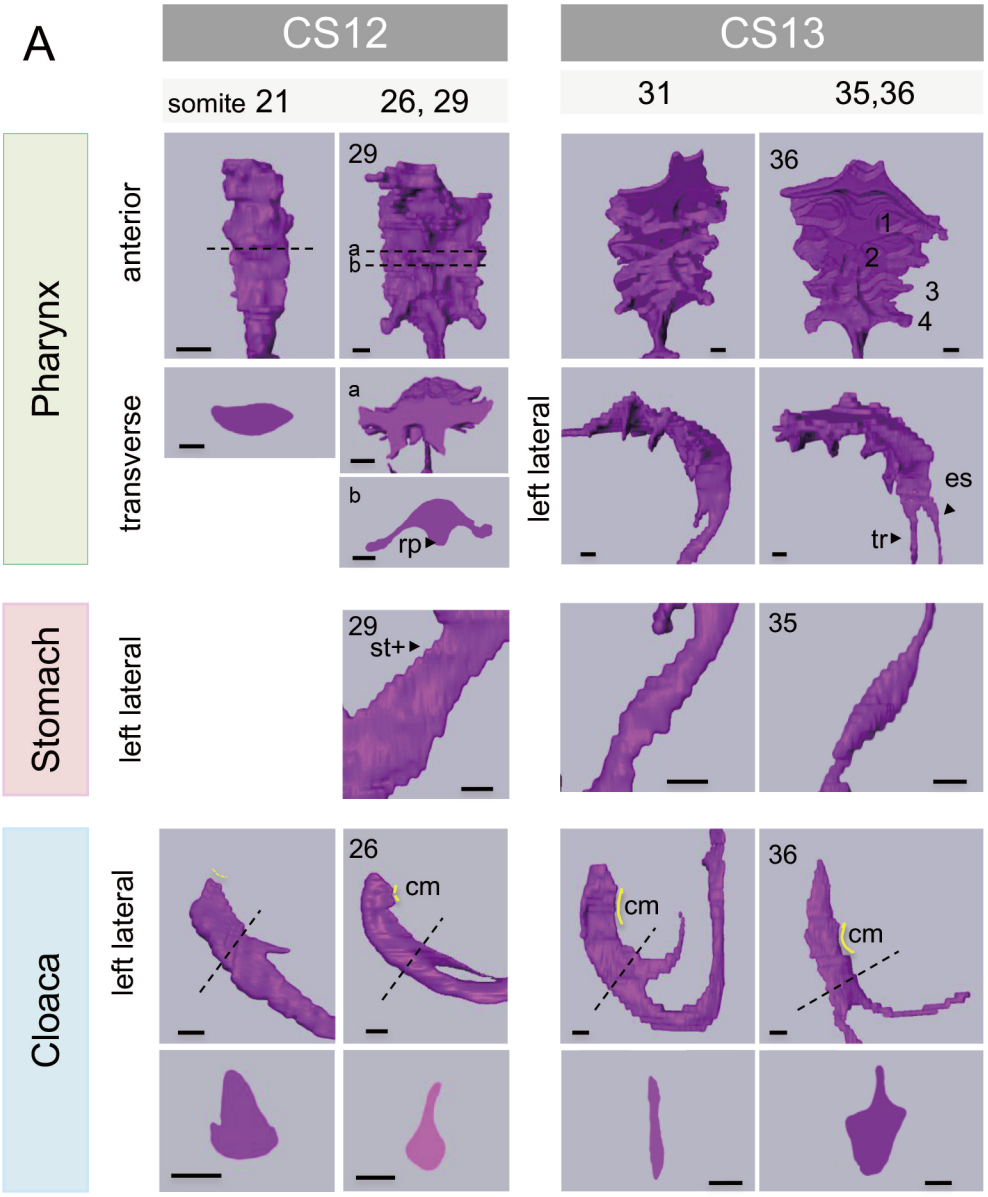
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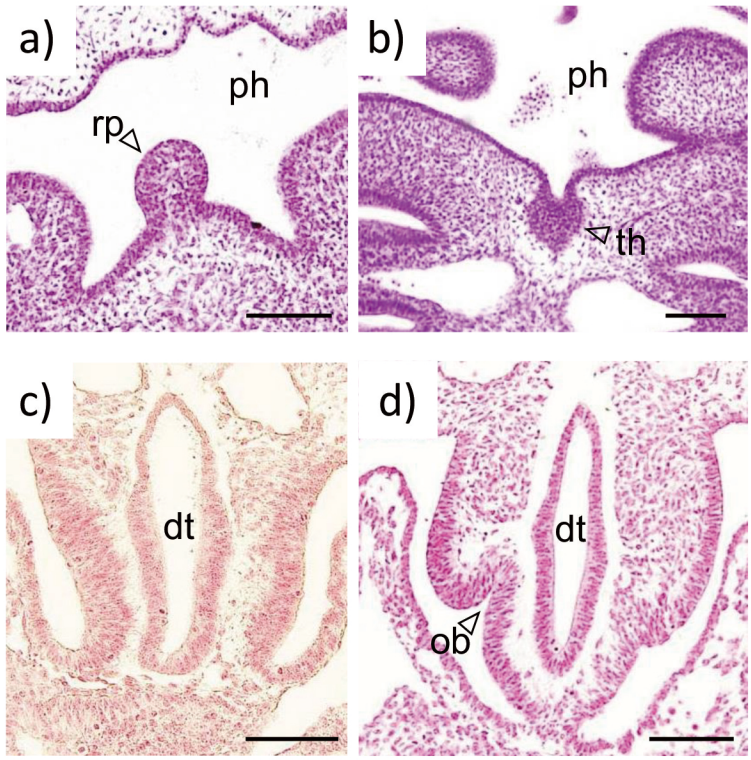

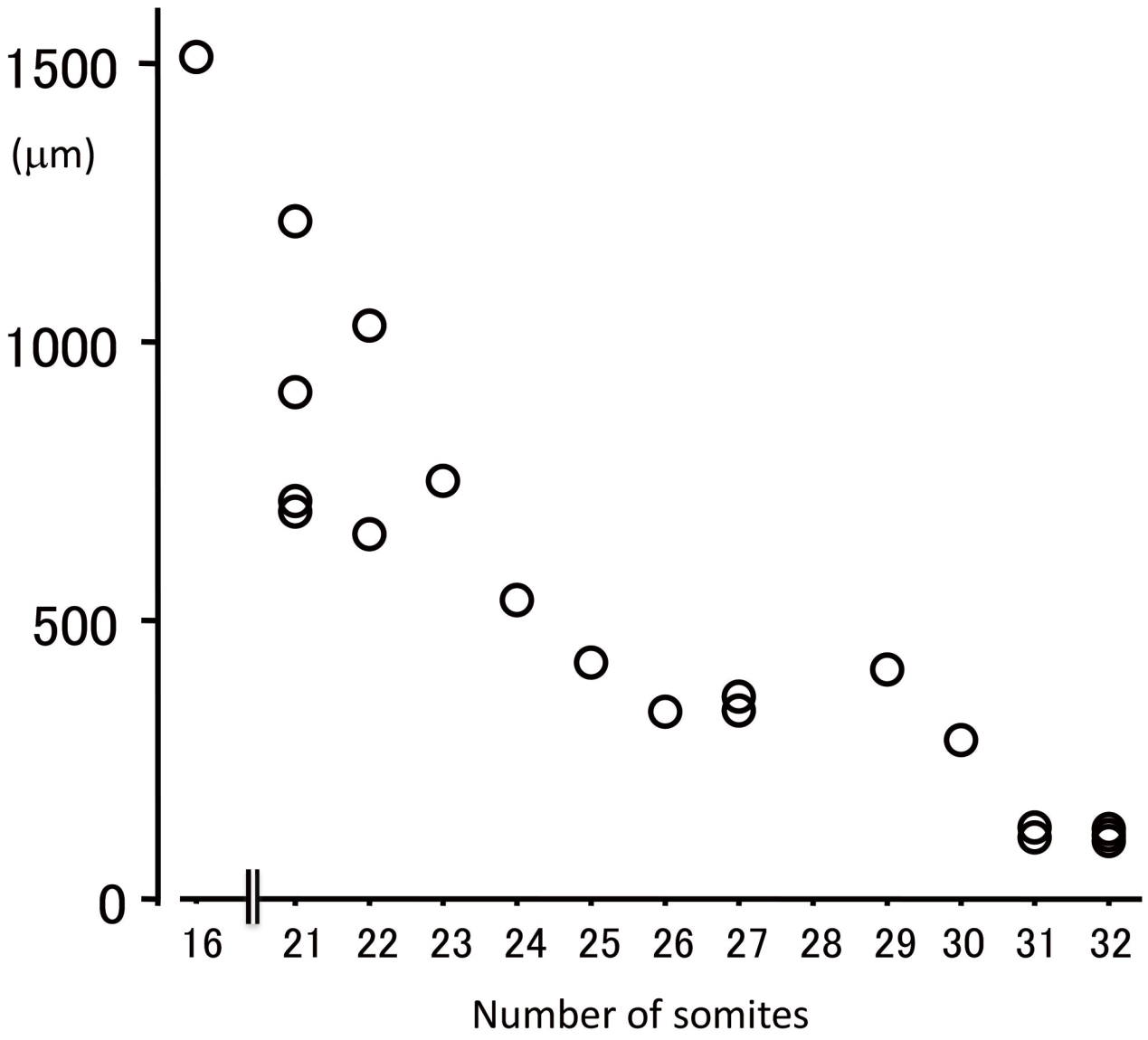
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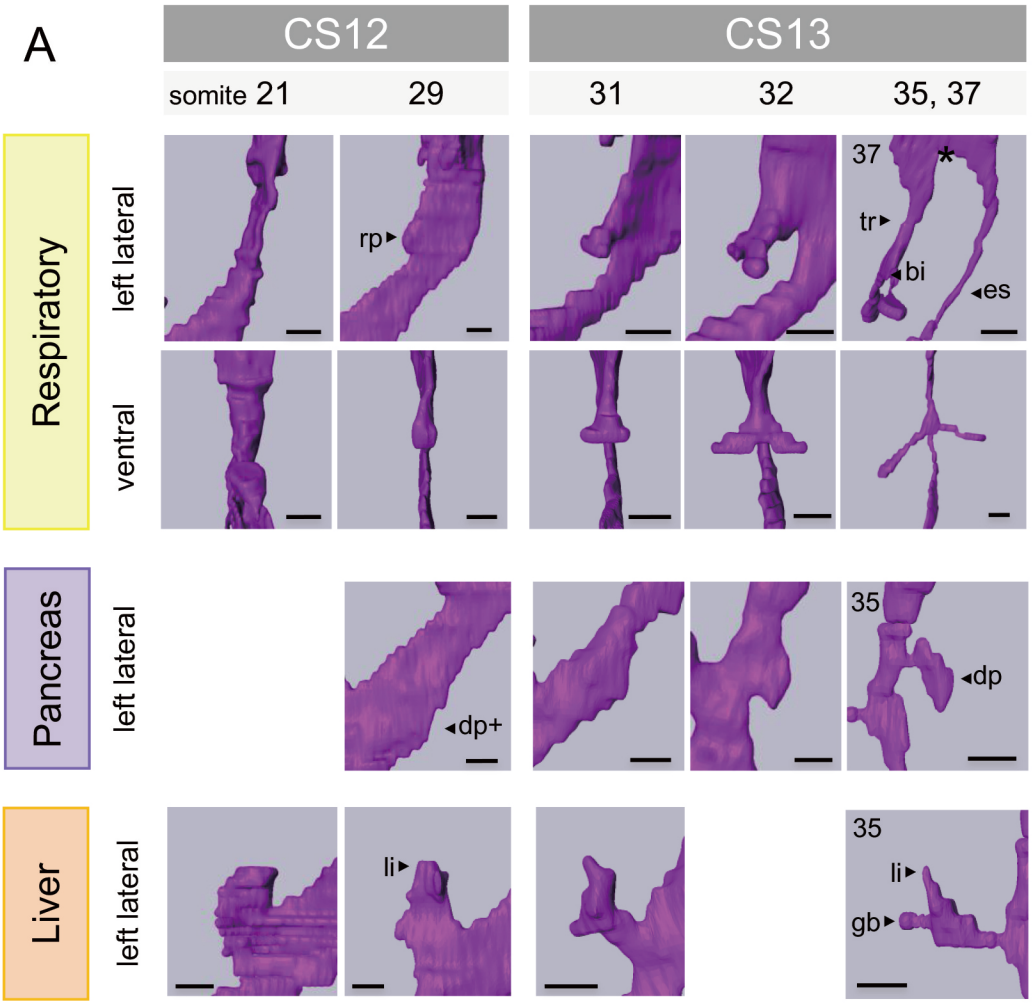
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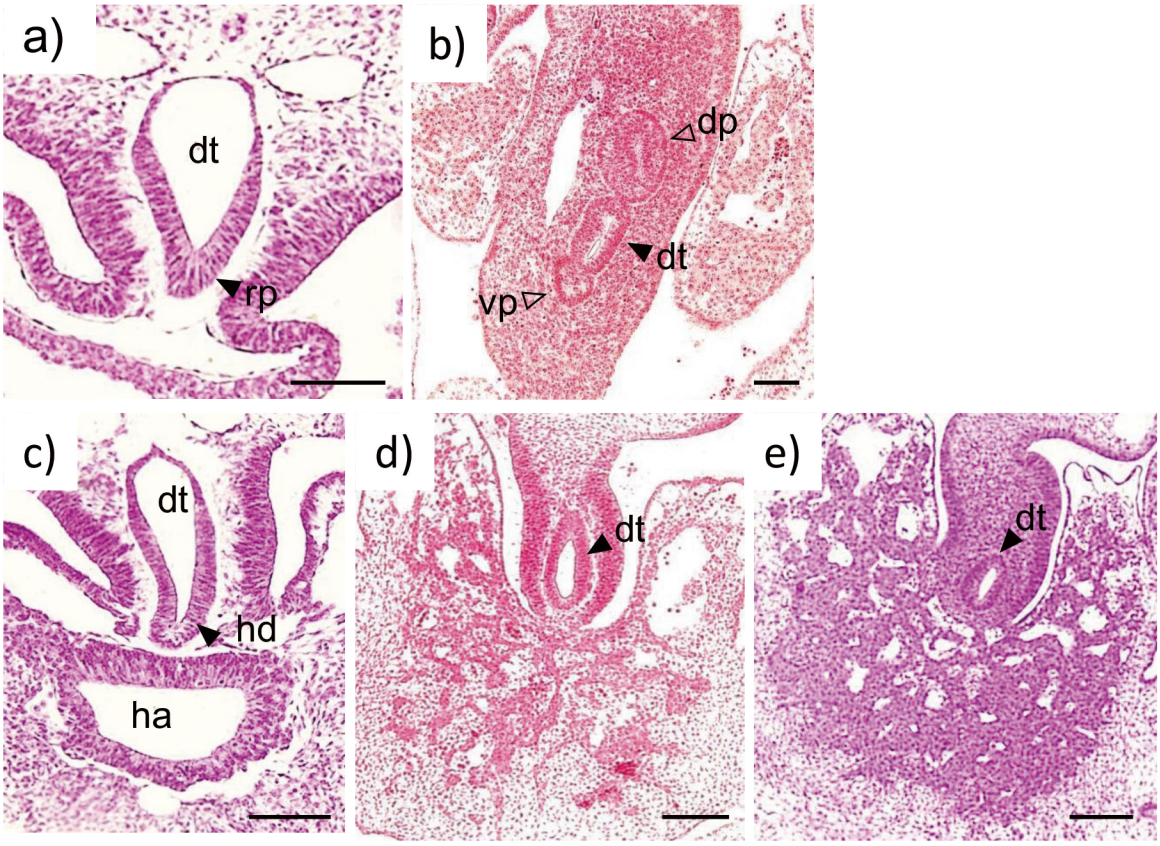
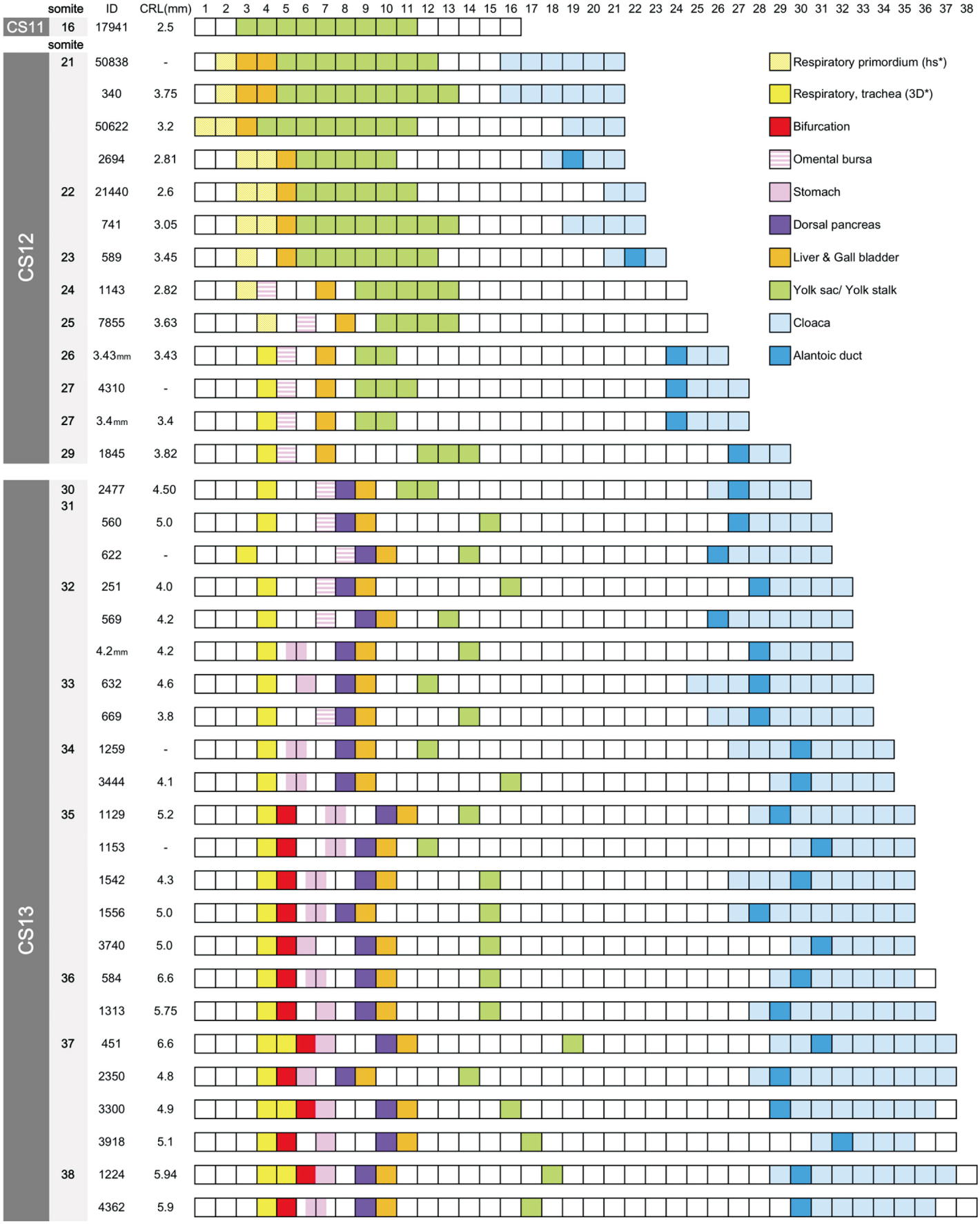
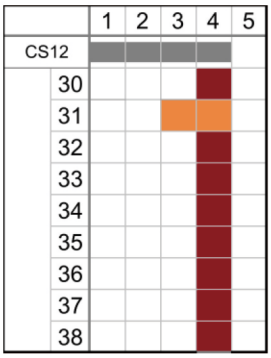
B

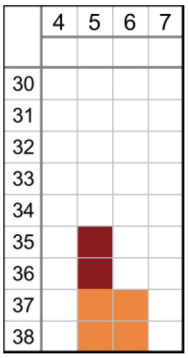


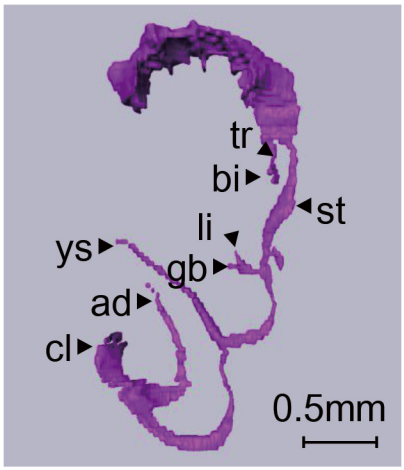

somite
ID
CRL(mm)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
CS11
16
17941
2.5
somite
CS12
21
50838
-
340
3.75
50622
3.2
2694
2.81
22
21440
2.6
741
3.05
23
589
3.45
24
1143
2.82
25
7855
3.63
26
3.43mm
3.43
27
4310
-
27
3.4mm
3.4
29
1845
3.82
CS13
30
2477
4.50
31
560
5.0
622
-
32
251
4.0
569
4.2
4.2mm
4.2
33
632
4.6
669
3.8
34
1259
-
3444
4.1
35
1129
5.2
1153
-
1542
4.3
1556
5.0
3740
5.0
36
584
6.6
1313
5.75
37
451
6.6
2350
4.8
3300
4.9
3918
5.1
38
1224
5.94
4362
5.9
Respiratory primordium (hs*)
Respiratory, trachea (3D*)
Bifurcation
Omental bursa
Stomach
Dorsal pancreas
Liver & Gall bladder
Yolk sac/ Yolk stalk
Cloaca
Alantoic duct

Trachea (tr)

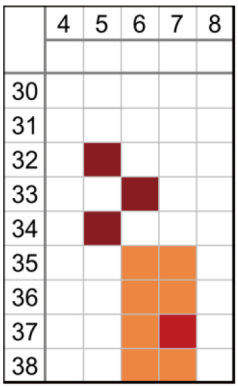


Bifurcation (bi)

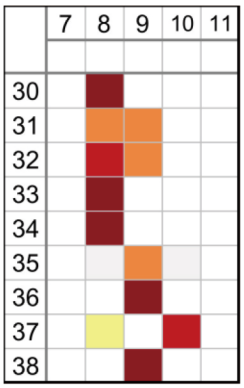




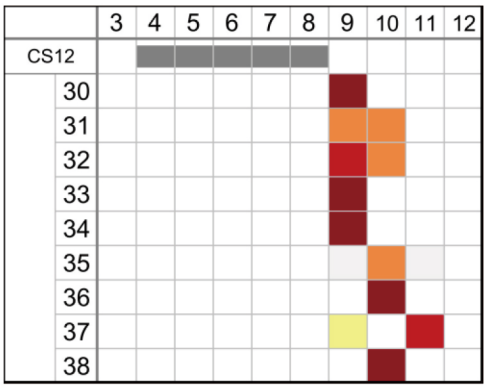
Stomach (st)

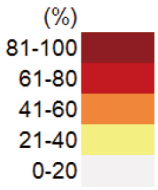


Dorsal pancreas (dp)

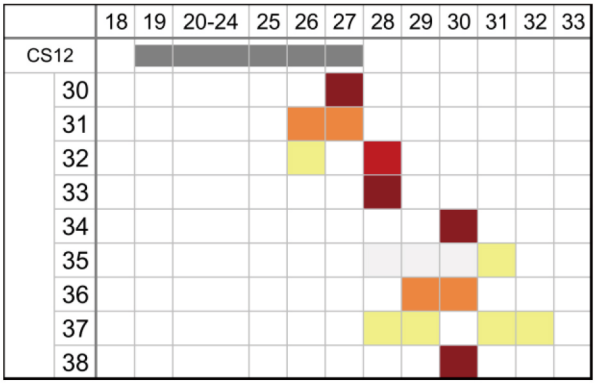


Liver (li)

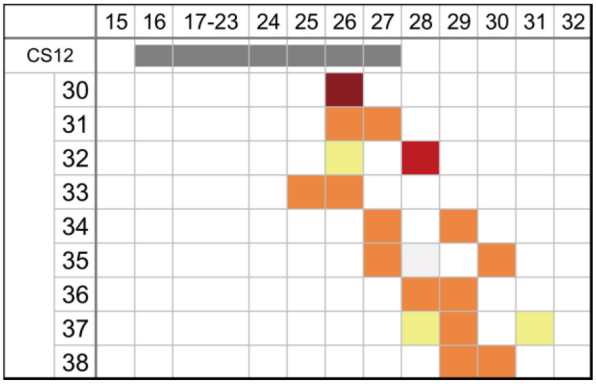


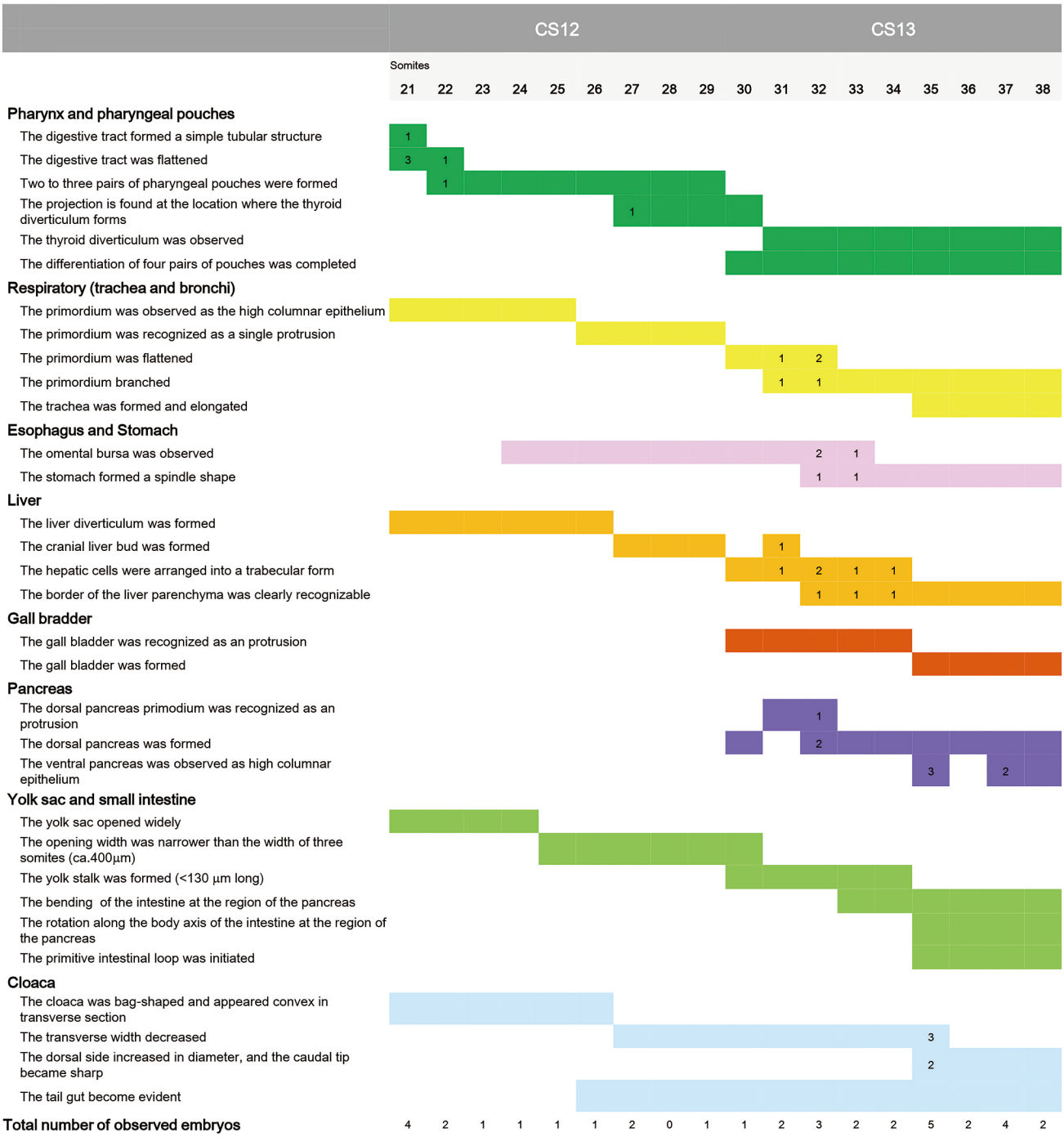


Allantoic duct (ad)



Cloaca (cl)




CS12
CS13
Somites
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
Pharynx and pharyngeal pouches
The digestive tract formed a simple tubular structure
The digestive tract was flattened
Two to three pairs of pharyngeal pouches were formed
The projection is found at the location where the thyroid diverticulum forms
The thyroid diverticulum was observed
The differentiation of four pairs of pouches was completed
Respiratory (trachea and bronchi)
The primordium was observed as the high columnar epithelium
The primordium was recognized as a single protrusion
The primordium was flattened
The primordium branched
The trachea was formed and elongated
Esophagus and Stomach
The omental bursa was observed
The stomach formed a spindle shape
Liver
The liver diverticulum was formed
The cranial liver bud was formed
The hepatic cells were arranged into a trabecular form
The border of the liver parenchyma was clearly recognizable
Gall bradder
The gall bladder was recognized as an protrusion
The gall bladder was formed
Pancreas
The dorsal pancreas primodium was recognized as an protrusion
The dorsal pancreas was formed
The ventral pancreas was observed as high columnar epithelium
Yolk sac and small intestine
The yolk sac opened widely
The opening width was narrower than the width of three somites (ca.400μm)
The yolk stalk was formed (<130 μm long)
The bending of the intestine at the region of the pancreas
The rotation along the body axis of the intestine at the region of the pancreas
The primitive intestinal loop was initiated
Cloaca
The cloaca was bag-shaped and appeared convex in transverse section
The transverse width decreased
The dorsal side increased in diameter, and the caudal tip became sharp
The tail gut become evident
Total number of observed embryos
4 2 1 1 1 1 2 0 1 1 2 3 2 2 5 2 4 2